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Sirolimus and Tacrolimus without Methotrexate as Graft-versus-Host Disease Prophylaxis after Matched Related Donor Peripheral Blood Stem Cell Transplantation

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ABSTRACT

Methotrexate in combination with a calcineurin inhibitor is a standard graft-versus-host disease (GVHD) prophylactic regimen in allogeneic stem cell transplantation. However, methotrexate is associated with delayed engraftment, mucositis, idiopathic pneumonia syndrome, and other transplant-related complications. Sirolimus, a novel immunosuppressant without methotrexate's toxicities, has been used successfully in solid organ transplantation. We hypothesized that replacing methotrexate with sirolimus would preserve effective prophylaxis of GVHD while minimizing transplant-related toxicity after allogeneic peripheral blood stem cell transplantation. We enrolled 30 patients in a phase II study to test the efficacy of tacrolimus in combination with sirolimus in lieu of methotrexate in preventing GVHD after allogeneic peripheral blood stem cell transplantation from HLA-matched related donors. Grade II GVHD occurred in 3 patients (10%), and no patient developed grade III or IV GVHD. Neutrophil and platelet engraftment were prompt, occurring on days 14 and 13, respectively. All patients survived to hospital discharge (median, 18 days), and peritransplantation toxicity was mild. Four patients developed thrombotic microangiopathy, and 3 patients developed hepatic veno-occlusive disease. Chronic GVHD occurred in 11 patients. Relapse-free and overall survival at 100 days were 93% and 97%, respectively, and were 71% and 67% at 1 year. Causes of death included relapse (n = 6), veno-occlusive disease (n = 1), and late pulmonary toxicity (n = 1). Sirolimus in combination with tacrolimus is a promising alternative to methotrexate-based regimens for GVHD prophylaxis after matched related donor peripheral blood stem cell transplantation. Mucositis was modest, engraftment was prompt, and transplant-related toxicity was modest. Methotrexate-free, sirolimus-based GVHD prophylactic regimens should be tested in randomized trials against the current standard of care.

KEY WORDS

Methotrexate • Peripheral blood stem cell transplantation • Sirolimus • Graft-versus-host disease

INTRODUCTION

Transplant-related toxicity and graft-versus-host disease (GVHD) are the 2 most critical barriers to successful allogeneic stem cell transplantation. The combination of a calcineurin inhibitor and methotrexate has been the standard GVHD prophylactic regimen for the past 20 years [1]. Despite these 2 agents, acute GVHD occurs after 35% to 40% of matched related donor transplantations, and transplant-related morbidity accounts for up to 25% of deaths in the posttransplantation period [2].

Sirolimus is a lipophilic macrocyclic lactone with antifungal and immunosuppressive properties. Structurally similar to tacrolimus, sirolimus complexes with FKBP12, binds mTOR, and blocks its function. The reported downstream effects of mTOR inhibition include blockade of CD28 co-stimulation signals [3],

selective transcription and translation blockade, and a decrease in the kinase activity of vital cyclins, ultimately leading to G_1 cell-cycle arrest [4]. In contrast, the tacrolimus/FKBP complex and the cyclosporin A/cyclophilin complex are immunosuppressive through the inhibition of calcineurin [5,6]. Although there is theoretical competition for FKBP binding sites between sirolimus and calcineurin inhibitors, these agents seem to work synergistically [5,6]. Sirolimus may also impair dendritic cell maturation [7] and antigen uptake [8,9] and may trigger dendritic cell apoptosis [10,11]. Sirolimus has been used alone and in combination with calcineurin inhibitors for the prevention of allograft rejection after solid organ transplantation [12,13] and as therapy for acute [14] and chronic [15,16] GVHD. The predominant toxicities of sirolimus are mild reversible cytopenia and hyperlipidemia with prolonged exposure. It is not associated with the neurotoxicity and nephrotoxicity of calcineurin inhibitors.

Methotrexate, used in conjunction with cyclosporine, provides additional control of GVHD [1]; however, it is associated with complications after transplantation. Methotrexate delays the time to neutrophil engraftment [1,17,18], worsens oral mucositis, and is associated with pulmonary toxicity after transplantation [19]. We therefore sought to replace methotrexate with an immunosuppressive agent without these limitations that could work synergistically with calcineurin inhibitors.

In a previous trial, we showed that the combination of sirolimus, tacrolimus, and low-dose methotrexate, when used after HLA-matched unrelated bone marrow transplantation, was associated with a rate of grade II to IV GVHD of 26% [20]. The GVHD rate after matched related donor transplantation is lower than that after unrelated donor transplantation; therefore, we believed that it would be safe to eliminate methotrexate from the GVHD prophylaxis regimen after matched related donor transplantation. We hypothesized that the use of sirolimus and tacrolimus would result in adequate control of GVHD and would reduce transplant-related morbidity and mortality.

METHODS

Study Design

This was a 1-stage, phase II trial of sirolimus and tacrolimus as GVHD prophylaxis for patients undergoing matched related donor allogeneic stem cell transplantation for hematologic malignancies. The primary end points of the trial were the incidence and severity of acute GVHD when sirolimus and tacrolimus are used in combination without additional methotrexate. Secondary end points included the incidence of serious complications after transplantation, the incidence and severity of mucositis after transplantation, the time to first hospital discharge, and survival at 100 days and 1 year after transplantation.

Eligible patients had HLA-matched related donors typed at high resolution for HLA class II and at intermediate resolution for HLA class I. Eligibility requirements included age >18 years, Eastern Cooperative Oncology Group performance status ≤2, and adequate measures of renal, hepatic, cardiac, and pulmonary function. The study was approved by the Office for the Protection of Research Subjects at the Dana-Farber Cancer Institute, and all participating subjects signed informed consent.

Study Therapy

Conditioning before transplantation consisted of cyclophosphamide (1800 mg/m^2) on 2 consecutive days followed by total body irradiation (14.0 Gy; 7 fractions). Lung shielding was used. Tacrolimus was administered at 0.02 mg/kg/d intravenously by continuous infusion beginning on day −3 (target serum concentration, 5-10 ng/mL). Tacrolimus was converted to an equivalent oral dose before discharge. Sirolimus was administered as a 12 mg oral loading dose on day −3, followed by a 4 mg/d single morning oral dose (target serum concentration, 3-12 ng/mL by high-performance liquid chromatography). Levels were monitored 3 times weekly while patients were hospitalized and then as clinically indicated after discharge. When clinically feasible, immunosuppressive therapy was tapered at day +100 after transplantation and was eliminated by week 26.

Peripheral blood stem cells were mobilized from related donors by using filgrastim (Amgen, Thousand Oaks, CA) at 10 μg/kg daily for 5 days. Stem cells were harvested by large-volume leukapheresis in 1 to 2 sessions to obtain a target stem cell dose of 5×10^6 CD34$^+$ cells per kilogram. The first day of stem cell infusion corresponded to day 0. No methotrexate was given after transplantation.

All patients were received posttransplantation supportive care as previously described [20]. Posttransplantation filgrastim was administered at 5 μg/kg/d from day +12 until neutrophil engraftment occurred. Acute GVHD was graded according to the consensus grading scale [21]. The incidence and severity of posttransplantation mucositis was prospectively recorded approximately twice weekly by trained oral evaluators using a validated 6-point site-specific mucositis scale based on the presence of erythema and extent of ulceration [22]. Chimerism was determined by single tandem repeat polymorphism analysis comparing posttransplantation samples with pretransplantation donor and recipient samples and with a 50% mixture of donor and recipient cells [23]. At monthly intervals

after transplantation, peripheral blood mononuclear cells were isolated by Ficoll-Hypaque sedimentation and incubated at 4°C for 30 minutes with a panel of monoclonal antibodies specific for CD3, CD4, CD8, CD20, CD45RO, CD45RA, and CD56 antigens conjugated to fluorescein, phycoerythrin, PC5, or PC7 (Coulter Immunology, Hialeah, FL). Immunophenotypic analysis of the stained cells was performed on a Cyomics FC500 (Beckman Coulter, Hialeah, FL).

Statistical Analysis

Sample size calculations were based on the assumption that an observed rate of GVHD <35% would be considered promising. The probability of concluding the proposed regimen promising is 0.84 if the true but unknown incidence rate is 30% and is 0.10 if the true but unknown incidence rate is 50%, with an exact binomial distribution.

Day 0 was considered the first day of stem cell infusion, regardless of the total number of infusions administered. Neutrophil and platelet engraftment were defined as the first of 3 consecutive days of an absolute neutrophil count of 500/μL or an unsupported platelet count of 20000/μL, respectively. Relapse-free survival was defined as the time from transplantation to relapse or death from any cause. Overall survival was defined as the time from transplantation to death from any cause. Patients alive without a relapse reported were censored at the date of last contact. Time to engraftment, time to hospital discharge, cumulative incidence of GVHD, relapse-free survival, and overall survival were calculated according to the method of Kaplan and Meier [24]. Locally weighted scatterplot smoothing, a smoothing curve estimation technique, was used to explore the pattern of longitudinal data on sirolimus levels [25].

RESULTS

Patient Characteristics

Thirty patients were enrolled between July 1, 2002, and May 15, 2003. A total of 86% of patients who underwent matched related donor transplantation during the enrollment period participated in the trial. The 5 patients who were excluded during this period were excluded because of prior stem cell transplantation (n = 2), preexisting hepatic insufficiency (n = 1), the need for stem cell cryopreservation (n = 1), and physician preference (n = 1).

Twenty-nine patients received HLA-matched peripheral blood stem cells from siblings. One patient received stem cells from a genotypically identical parent. Patient characteristics are shown in Table 1. Adequate serum concentrations of sirolimus, when given orally, were attainable in all patients (Figure 1). Targeted sirolimus levels were noted in 73.3% of measured values between days 0 and 100. Of the remaining measurements, 14.4% were subtherapeutic (<3 ng/mL), and 12.3% were supratherapeutic (>12 ng/mL).

Table 1. *Baseline Characteristics*

Variable	Data
Sample size	30
Age, y, median (range)	42 (19-54)
Sex	
Male	16 (53%)
Female	14 (47%)
Stem cell source	
PBSC	30 (100%)
Donor	
6/6 HLA-matched sibling	29 (97%)
6/6 HLA-matched parent	1 (3%)
Diagnosis	
AML	8 (27%)
CR1	3
CR > 1	2
Refractory/active disease	3
MDS	7 (23%)
Untreated RA/RARS	4
RAEB/RAEB-t	3
CML	7 (23%)
Chronic phase	6
Advanced	1
NHL	6 (20%)
Follicular	1
Diffuse large B cell	1
Burkitt lymphoma	1
CLL/SLL	1
Mantle cell lymphoma	1
Peripheral T-cell lymphoma	1
ALL	1 (3%)
Second remission	1
ATLL	1 (3%)
Donor age, y, median (range)	42 (16-59)
Sex matching (donor/recipient)	
M/M	7 (23%)
M/F	6 (20%)
F/M	9 (30%)
F/F	8 (27%)
CMV serostatus (donor/recipient)	
−/−	9 (30%)
−/+	6 (20%)
+/−	2 (7%)
+/+	10 (33%)
Unknown	3 (10%)

PBSC indicates peripheral blood stem cells; AML, acute myelogenous leukemia; MDS, myelodysplastic syndrome; CML, chronic myelogenous leukemia; NHL, non-Hodgkin lymphoma, ALL, acute lymphoblastic lymphoma; ATLL, acute T-cell leukemia/lymphoma; CR, complete remission; RA/RARS, refractory anemia/refractory anemia with ringed sideroblasts; RAEB/RAEB-t, refractory anemia with excess blasts/refractory anemia with excess blasts in transformation; M, male; F, female; CLL, chronic lymphatic leukemia; SLL, small lymphocytic lymphoma.

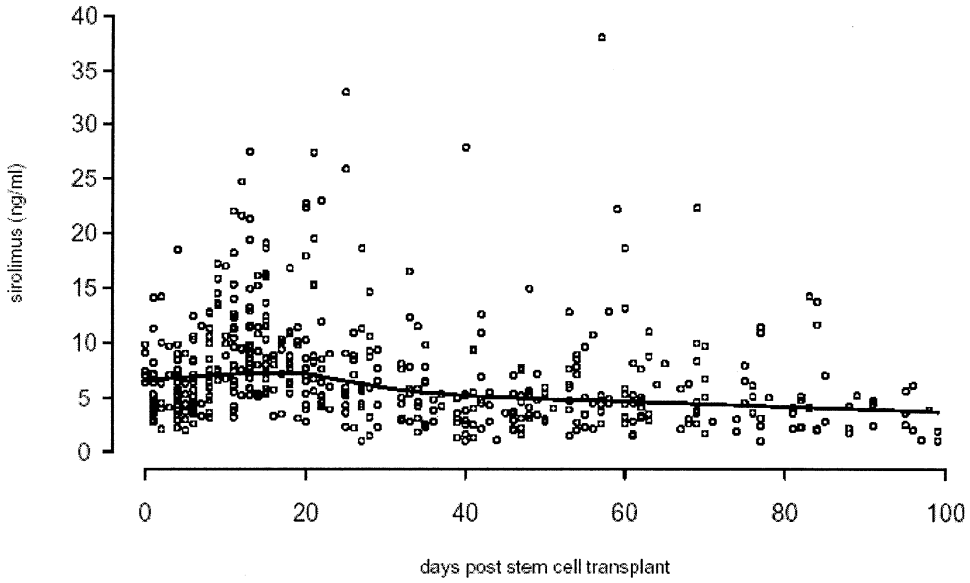

Figure 1. Sirolimus levels during 100 days after transplantation.

Stem Cell Products and Engraftment

The median number of $CD34^+$ stem cells infused was 7.83×10^6/kg (range, 2.81-20.60 $\times$ 10^6/kg). Nine patients received more than 1×10^7 cells per kilogram, and 1 patient received fewer than 3×10^6 $CD34^+$ cells per kilogram. All patients achieved stable hematopoietic engraftment. The median time to neutrophil engraftment was 14 days (range, 1-17 days). The median times to attain a platelet count of 20000 and 100000/μL were 13 days (range, 10-47 days) and 18 days (range, 11-189 days), respectively. Two patients relapsed before achieving a platelet count of 100000/μL. All 30 patients survived to their first hospital discharge, which occurred at a median of 18 days (range, 15-54 days) from transplantation (Table 2; Figure 2). Complete donor hematopoietic chimerism was noted in all samples tested within 100 days of transplantation. Reconstitution of B, T, and NK cells was notable for a decrease in the total number of $CD8^+$ T cells early after transplantation (Figure 3).

Table 2. *Engraftment and GVHD End Points*

Variable	Data
Median days to ANC >500/μL (range)	**14 (11-17)**
Median days to Plt >20 000/μL (range)	**13 (10-47)**
Median days to Plt >100 000/μL (range)*	**19 (11-189)**
Median days to first hospital discharge	**18 (15-54)**
Acute GVHD	
Grade 0-I	**27 (90%)**
Grade II	**3 (10%)**
Grade III-IV	**0 (0%)**
Chronic GVHD†	**11 (39%)**

ANC indicates absolute neutrophil count; Plt, platelets.

*Two patients relapsed before achieving a platelet count of 100 000/μL.

†Two patients died before day 100 and were ineligible for chronic GVHD analysis.

Graft-versus-Host Disease

Three patients developed grade II acute GVHD at 11, 20, and 21 days from transplantation. The actuarial incidence of grade II acute GVHD was 10% at 100 days (Figure 4). No patient developed grade III or IV GVHD. All 3 patients with grade II GVHD had cutaneous GVHD (maximum skin stage III), and 1 patient also had gastrointestinal involvement (maximum gastrointestinal stage I). No patient developed hepatic GVHD. In total, 5 patients received systemic therapy for grade I or II GVHD with corticosteroids, with or without daclizumab (1 mg/kg), as part of a blinded randomized trial examining initial therapy for acute GVHD. One additional patient received low-dose corticosteroids for therapy of intractable nausea after upper gastrointestinal endoscopy failed to document histologic changes consistent with GVHD.

Chronic GVHD was reported in 11 of 28 evalu-

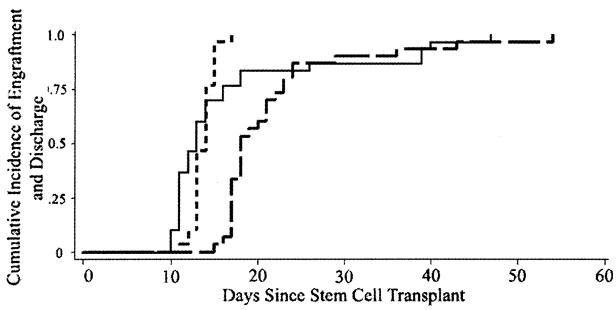

Figure 2. Neutrophil and platelet engraftment and time to first hospital discharge. Neutrophil (- - -), discharge (– –), platelet (——).

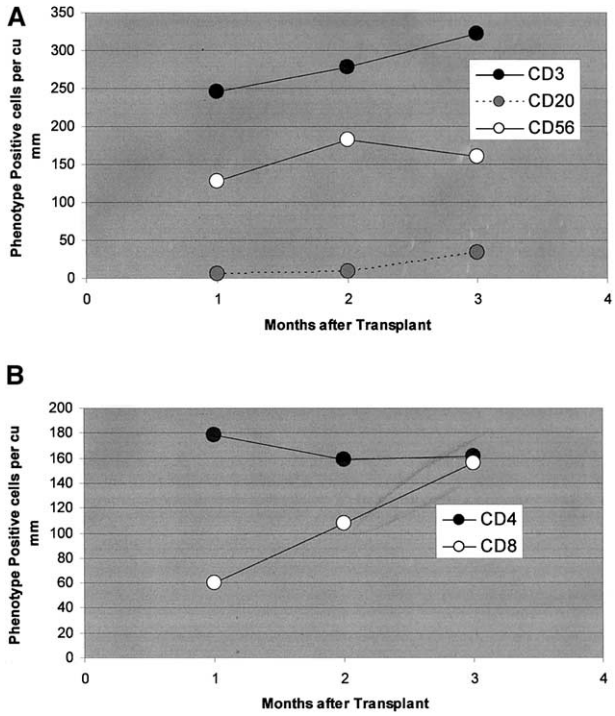

Figure 3. Immunologic reconstitution after transplantation. A, CD20 B cell, CD3 T cell and CD56 NK cell reconstitution. B, T cell subset (CD4, CD8) reconstitution.

able patients. Five patients had chronic GVHD limited to mucocutaneous organs, 4 had hepatic involvement, and 2 had other organ involvement. The median time to development of chronic GVHD was 218 days. Chronic GVHD occurred in 6 patients during a taper of immunosuppression and in 5 patients who were receiving no immunosuppressive therapy. Two of the 3 patients with grade II acute GVHD developed chronic GVHD. There have been no deaths attributable to acute or chronic GVHD.

Toxicity

Regimen-related toxicity was moderate and is shown in Table 3. No patient developed idiopathic pneumonia syndrome or diffuse alveolar hemorrhage. Three patients developed severe veno-occlusive disease of the liver (VOD). Two of these patients had

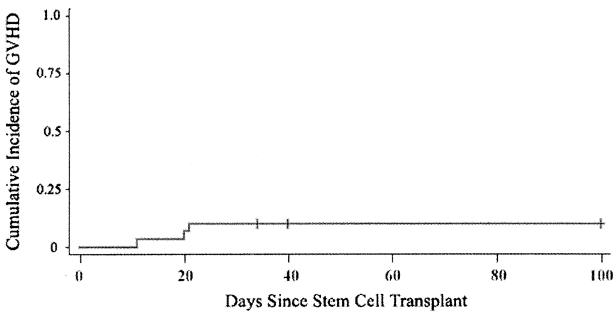

Figure 4. Cumulative incidence of grade II to IV GVHD.

Table 3. *Transplant-Related Toxicity*

Variable	Data
Idiopathic pneumonia syndrome	0 (0%)
Veno-occlusive disease of the liver	3 (10%)
Thrombotic microangiopathy	4 (13%)
CMV reactivation	1 (3%)
BK cystitis/viremia	1 (3%)
Invasive fungal infection	0 (0%)
Hypercholesterolemia	
CTC grade 2	3 (10%)
CTC grade 3	0 (0%)
CTC grade 4	0 (0%)
Hypertriglyceridemia	
CTC grade 2	1 (3%)
CTC grade 3	1 (3%)
CTC grade 4	1 (3%)

CTC indicates common toxicity criteria.

previously been exposed to gemtuzumab ozogamycin, a known risk factor for VOD [26]. All 3 patients were treated with the experimental agent defibrotide [27]. VOD was the cause of death in 1 patient, and the other 2 patients recovered normal hepatic function.

The syndrome of thrombotic microangiopathy (TMA), which includes renal dysfunction, microangiopathic hemolysis, and thrombocytopenia, occurred in 4 patients (13%). As a result of TMA, 1 patient required temporary hemodialysis. All patients with TMA regained normal renal function. Before TMA diagnosis, supratherapeutic serum tacrolimus and sirolimus levels were noted in 2 and 3 patients, respectively. TMA was managed conservatively by discontinuing tacrolimus and withholding sirolimus until renal function improved and serum levels fell into the target range. Temporary hemodialysis was required in 1 patient. All 4 patients regained normal renal function. Mycophenolate mofetil was substituted for tacrolimus in all patients with TMA. No patient experienced a recurrence when sirolimus was reintroduced.

Reactivation of cytomegalovirus (CMV) was noted in only 1 patient despite 18 CMV-seropositive donor/recipient pairs at the time of transplantation. One patient developed BK cystitis and viremia and was treated with supportive care. No other patient developed an opportunistic infection or invasive fungal disease.

Oral mucositis was modest. Ten patients (33%) developed nonulcerating mucositis (grade 0-1), and only 3 patients (10%) developed severe (grade 4) mucositis. No patient developed grade 5 mucositis. As a result, the median number of days on total parenteral nutrition was 6 (range, 0-26 days), and 47% of patients required no parenteral nutrition during transplantation. Hypercholesterolemia and hypertriglyceridemia were noted in 3 patients each. Two patients experienced both hypercholesterolemia and hypertriglyceridemia,

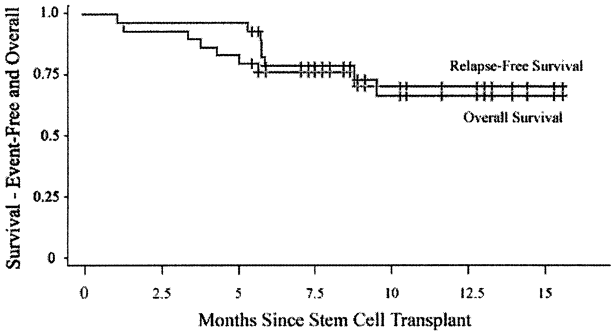

Figure 5. Relapse-free and overall survival.

1 patient experienced isolated hypercholesterolemia, and 1 patient experienced isolated hypertriglyceridemia as a result of prolonged exposure to sirolimus. These 4 patients were treated with 3-hydroxy-3-methylglutaryl/coenzyme A inhibitors.

Survival and Outcome

The median follow-up of transplant patients was 262 days, with a minimum follow-up of 6 months among surviving patients. Nonrelapse mortality was 3% at 100 days and was 6% at 1 year (1 case of VOD and late pulmonary toxicity). Six patients with high-risk disease at the time of transplantation relapsed and died of relapsed disease. All other patients remain alive in complete clinical remission. Relapse-free and overall survival at 100 days were 93% and 97%. At 1 year, relapse-free and overall survival were 71% and 67%, respectively (Figure 5).

DISCUSSION

Prevention of acute GVHD and minimization of transplant-related morbidity and mortality remain critical barriers to improved outcomes after matched related peripheral blood stem cell transplantation. We hypothesized that methotrexate, an antiproliferative agent commonly used for GVHD prophylaxis, adversely affects transplantation outcomes and that replacement of methotrexate with sirolimus would reduce GVHD and minimize transplant-related toxicity.

The expected reduction in transplant-related toxicity was evident. This reduction, hypothesized to be due to the omission of methotrexate, could have resulted from several factors. The first is the reduction in the time to hematopoietic engraftment. The neutropenic period, during which the incidence of infectious, hemorrhagic, and other complications is greatest, was reduced by several days. The median time to attain a neutrophil count of 500/μL in this study was 14 days, which compares favorably to that in recent randomized studies, in which the median time to neutrophil engraftment varied from 16 to 19 days when methotrexate and a calcineurin inhibitor were used [28,29]. This is consistent with prior studies of transplantation that compared methotrexate-containing and non–methotrexate-containing regimens [1,30].

The second mechanism possibly responsible for the reduction in transplant-related toxicity is the absence of methotrexate-induced tissue injury. Idiopathic pneumonia syndrome and diffuse alveolar hemorrhage, which have been associated with methotrexate use [19] were not noted in any treated patients. VOD of the liver occurred in only 1 patient not previously exposed to gemtuzumab ozogamycin. In comparison, the incidence of these 2 important causes of morbidity and mortality after stem cell transplantation varies from 3% to 15% for idiopathic pneumonia syndrome [31] and from 10% to 60% for VOD of the liver [32]. Mucositis is a cause of significant morbidity and reduction in quality of life after hematopoietic stem cell transplantation, and methotrexate, when given in conventional dosing regimens, is an important contributor to this problem [33,34]. Attempts to reduce the incidence and severity of oral mucositis after transplantation have been largely unsuccessful [33,35]. It is likely that the omission of methotrexate in this study led to the reduction in observed mucositis as well.

Previous attempts to eliminate or replace methotrexate in GVHD regimens have been unsuccessful. In randomized studies in which methotrexate was omitted or replaced with corticosteroids, acute [1,30,36,37] and chronic [38,39] GVHD rates were higher. The 4-dose methotrexate regimen (45 mg/m^2 total) is most commonly used after transplantation; however, alternative and abbreviated regimens exist [40-45], and the optimal regimen is unknown. Grade II to IV acute GVHD rates have not been shown to be increased in retrospective studies in which less than the standard 4-dose methotrexate regimen was administered [18,46,47]. Furthermore, a single study of tacrolimus monotherapy noted no difference in the incidence of acute GVHD after bone marrow transplantation [48], although monotherapy with tacrolimus was insufficient when peripheral blood stem cells were used [49]. We demonstrated that substitution of sirolimus for methotrexate was more effective in preventing grade II to IV acute GVHD in comparison with historical controls [28,29,45], with an actuarial rate of only 10% at 100 days. This low incidence of GVHD was noted even as peripheral blood stem cells, whose use may be associated with increased rates of acute GVHD, were used [50].

Methotrexate functions primarily by killing antigen-activated T cells; however, methotrexate causes tissue damage and can activate the initial phase of the GVHD response [51,52]. Sirolimus has multiple immunosuppressive effects, including impairment of antigen uptake by dendritic cells [8,9], and may therefore block the initiating and propagating events of GVHD.

In addition, proapoptotic signaling in dendritic cells induced by sirolimus [11] may minimize the potential of dendritic cells to be potent stimulators of alloimmunity. Sirolimus specifically inhibits $CD8^+$ T-cell proliferation [53]; this effect was noted in our trial, with an inverted CD4/CD8 reconstitution ratio when compared with other studies of immune reconstitution [54]. Because $CD8^+$ T cells are important mediators of GVHD [54], this may have contributed to improved GVHD control. Other contributing factors to the excellent GVHD control include the prolonged course of dual immunosuppressive therapy and the fact that the combination of sirolimus and tacrolimus may be more synergistic than combinations of sirolimus and other calcineurin inhibitors [55].

The absence of GVHD in this trial was not correlated with profound immunosuppression and poor engraftment, because all patients developed complete donor chimerism by day 100. Similarly, the absence of a graft-versus-malignancy effect has not been noted, because only patients with high-risk disease (relapsed or refractory acute myelogenous leukemia, relapsed acute lymphoblastic lymphoma, and Burkitt non-Hodgkin lymphoma) have relapsed. Longer follow-up will be necessary to definitively comment on relapse rates. Finally, the incidence of opportunistic infections was low, with no invasive fungal infections, only 1 case of CMV reactivation, and only 1 case of BK virus–related disease. Although other members of the sirolimus family (such as RAD001 [everolimus]) may have antiviral properties, it is unclear whether this mechanism of action played a role in the low incidence of viral reactivation in this trial. More likely is the possibility that in the absence of acute GVHD, CMV reactivation was suppressed.

Sirolimus and tacrolimus led to an apparent increase in the rate of TMA in this study. The association of TMA with cyclosporine or tacrolimus after stem cell transplantation and solid organ transplantation is well known [56]. Recently, the combination of sirolimus and calcineurin inhibitors has been associated with this syndrome [57]. Sirolimus may promote TMA via direct endothelial damage or may potentiate the effects of calcineurin inhibitors [58,59]; however, there is only 1 case report of de novo TMA related to sirolimus monotherapy [60]. The mechanism responsible for sirolimus-induced microangiopathy may be molecular mimicry between sirolimus and tacrolimus, but conversion from tacrolimus to sirolimus immunosuppression as therapy for TMA without apparent untoward effects has been reported [61-63]. We suggest strict monitoring of tacrolimus and sirolimus levels when these drugs are used in combination to prevent TMA.

In summary, we have shown that the substitution of methotrexate with sirolimus is safe and effective for the prevention of acute GVHD. This regimen is associated with prompt engraftment, minimal transplant-related morbidity and mortality, and minimal mucositis. Sirolimus-containing, methotrexate-free immunosuppressive regimens offer the possibility of a major advancement in GVHD prophylaxis and should be tested against standard methotrexate regimens. If a randomized trial against a standard methotrexate regimen confirms our promising findings, then the sirolimus/tacrolimus combination would be the first major modification of GVHD prophylaxis in 20 years.

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